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Substrate Binding to Cytochromes P450

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Abstract

P450s have attracted tremendous attention due not only to their involvement in the metabolism of drug molecules and endogenous substrates but also the unusual nature of the reaction they catalyze, namely the oxidation of unactivated C-H bonds. The binding of substrates to P450s, which is usually viewed as the first step in the catalytic cycle, has been studied extensively via a variety of biochemical and biophysical approaches. These studies were directed towards answering different questions related to P450s including, mechanism of oxidation, substrate properties, unusual substrate oxidation kinetics, function, and active site features. Some of the substrate binding studies extending over a period of more than forty years of dedicated work has been summarized in this review and categorized by the techniques employed in the binding studies.

Keywords

Substrate binding; cytochrome P450; cooperativity; ligands; drug development

Introduction

Cytochrome P450s are heme-containing enzymes catalyzing the mixed-function oxidation of endogenous substrates, as well as xenobiotic molecules [1-4]. P450s are found in most living systems, from bacteria to humans, with more than 8500 P450 genes reported to date (drnelson.utmem.edu/CytochromeP450.html) [5, 6]. Earlier studies had focused on bacterial P450s with particular emphasis on camphor-oxidizing P450 101A1 ($P450_{cam}$) from *Pseudomonas putida* [7-9, 10], laying the groundwork for understanding the catalytic cycle in other P450s [11, 12]. Sequencing of the human genome showed the presence of 57 human P450 genes and 58 pseudogenes (drnelson.utmem.edu/CytochromeP450.html). Endogenous substrates, as well as the biological function of majority of the human P450 enzymes, have been well-characterized [13] whereas the functional properties of some human P450s (so-called "orphans") are not known [14]. In addition to the oxidation of endogenous substrates,

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human P450s are involved in the metabolism of 75% of the drugs on the market, 95% of which are oxidized by P450s 3A4, 2D6, 2C9, 2C19 and 1A2 [4, 15, 16]. Mainly due to these diverse substrate properties (from ethylene with M_r 28 to cyclosporine with M_r 1201) and the critical impact on drug development and metabolism-mediated toxicity, significant research efforts have focused on the study of the human P450s in the last twenty years [13, 17].

Substrate binding in the P450 catalytic cycle

The P450 catalytic cycle leading to substrate oxidation is rather complex (Fig. 1) and has been investigated in detail (for recent reviews see [12, 18, 19]). Although the substrate binding step is depicted as taking place prior to the reduction of the heme iron via the electron provided by NAPDH-P450 reductase, it has been shown that the substrate may bind and/or dissociate at other steps of the catalytic cycle as well [20, 21]. Binding of substrate leads to displacement of water as the sixth ligand to the heme iron, changing the spin state of the iron from low- to high-spin [22]. The spin state change in turn increases the oxidation-reduction potential ($E_{m,7}$) (facilitating the reduction for thermodynamic reasons) for some bacterial P450s, in particular P450 101A1 ($P450_{cam}$) [8, 23]. A similar observation has been reported for bacterial P450 102A1 ($P450_{BM3}$) [24] whereas no change in the heme redox potential was detected for bacterial P450 176A1 ($P450_{cin}$) [31]. The effect of substrate binding on the heme oxidation-reduction potential and rates of P450 reduction seem to be more complicated for mammalian P450s [25-30]. . The effect of substrate binding is related to the relative affinity of the substrate for the ferric and ferrous forms of the enzyme [32]; i.e. tighter binding to the reduced form of the enzyme raises the $E_{m,7}$. In a recent study using P450 3A4 Nanodiscs, an increase in the $E_{m,7}$ of 80 mV has been reported upon binding of substrates, accompanied by a change in the spin state [33]. This change did not occur, however, in a study with purified P450 3A4 in a system composed of a phospholipid mixture and apo-cytochrome b_5 [29]. Regardless of the effect on the reduction potential, studies in this laboratory have shown that the rate of reduction of ferric forms of P450 3A4, 2A6, 2C9, and 2C19 is stimulated in the presence of substrates [20, 30, 34, 35] whereas the presence of substrate did not have any effect on the reduction rate of ferric P450 1A2 or 2E1 [30]. The kinetics of reduction are not strictly linked to the thermodynamic ease of reduction.

Substrate binding studies

Interaction of substrates with enzymes have been traditionally viewed as a key-lock type of phenomena, where a particular substrate binds to a designated part of the enzyme (active site) to allow catalysis to take place. While this general understanding may be true for some P450-substrate interactions [20, 36, 37], recent studies using a variety of experimental approaches have demonstrated that the situation is more complex than a stoichiometric one-step, two-state substrate-enzyme interaction [21, 38-42]. Indeed, x-ray crystal structures of P450s obtained for some bacterial as well as mammalian forms are consistent with multiple occupancy of P450 active sites, with bacterial P450 107A1 ($P450_{eryF}$) [43], P450 158A1 [44] and P450 158A2 [45] from the actinomycete *Streptomyces coelicolor* A3(2), and—of particular interest—human P450 3A4 [46] (for recent reviews of structural features of

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mammalian P450s see [47, 48] and [49]). Considering the structural diversity of the molecules oxidized by P450s (Fig. 2), unusual substrate-P450 interactions are not that surprising.

The study of substrate binding by P450s is of interest due to many reasons including the investigation of substrate specificity of P450s, unusual substrate oxidation kinetics (i.e. cooperativity), active site features of P450s, understanding the individual steps of the P450 catalytic cycle (i.e. investigating the subsequent steps and intermediates involved), and the understanding of P450 function. A vast array of approaches has been utilized to investigate both the kinetics and thermodynamics of substrate-P450 interactions. In this review, a selection of studies are classified and presented according to the analytical approach used.

Absorbance spectroscopy

Binding of endogenous substrates and xenobiotics (Fig. 2) to P450s results in two types of characteristic spectral changes in the UV-visible heme Soret spectrum, referred to as Type I and Type II [22, 50, 51]. Displacement of water as the sixth ligand to heme iron results in a peak at ~390 nm accompanied by a trough at ~418 nm nm giving rise to a Type I shift in the difference spectrum (Fig. 3). Direct coordination of a ligand to heme iron results in a Type II shift characterized by a shift to 430-455 nm, but these complexes are inhibitory and generally not considered relevant to productive substrate binding leading to catalysis. These spectral changes have been extremely useful in studying binding of substrate to P450s and steady-state titration of a P450 (with increasing concentrations of the ligand of interest) has been used to estimate spectral dissociation constants (K_d or K_s) [52], an indication of binding affinity or the total binding energy (ΔG_{bind}) [53]. Substrate binding to P450 3A4, in particular, has been studied extensively [40, 54] in an attempt to explain the unusual oxidation kinetics observed with this enzyme [54-56] and its dominant involvement in the oxidation of drug molecules [15, 57]. Cooperative binding of testosterone to P450 3A4 has been shown in binding titrations, as indicated by the sigmoidal dependence of the low- to high-spin shift on testosterone concentration (with a Hill coefficient of $n = 1.3$) [34, 58]. These results suggest the possibility that two interacting binding sites exist for testosterone. Later, the presence of a second binding site was demonstrated indirectly using morphiceptin, a Type II ligand, which seems to be independent from the testosterone binding site but overlaps with the αNF binding site [34].

Roberts *et al.* studied testosterone binding to P450 3A4 via EPR and optical titrations carried out at P450 concentrations both higher and lower than the dissociation constant [59]. The authors concluded that the affinity for the binding of a second testosterone molecule has a lower affinity than the first one (negative cooperativity in binding affinity). However, the low- to high-spin state change was considered to be less efficient for the first testosterone molecule compared to the second one (positive cooperativity in spin state change) [59]. A similar positive cooperativity has been reported for caffeine binding to P450 3A4 as well [60]. It was also shown that the affinity for the testosterone binding increases in the presence of one equivalent of αNF, suggesting heterotropic cooperativity [38]. Furthermore, the first molecule of αNF is proposed to bind at a peripheral site because this binding interaction does not lead to a significant change in the heme spin state. Application of a

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spectrophotometric "titration-by-dilution" approach [61] has also been used to estimate binding stoichiometry to P450 3A4. In this work, Job titrations [62, 63] revealed that one molecule of bromocriptine binds per molecule of P450 3A4, whereas 1-pyrenebutanol seems to bind with 2:1 (ligand:P450) stoichiometry and with a lower affinity for the second binding site than the first, consistent with the earlier work with testosterone [42]. Substrate binding to P450 3A4 incorporated into Nanodiscs, which serve as a nanoscale phospholipid bilayer yielding to a monomeric solubilized form of the enzyme, has been studied in detail [64, 65]. In the absence as well as co-incorporation of NADPH-P450 reductase into Nanodiscs, testosterone binding showed sigmoidal binding curves with a reported Hill coefficient of 1.6 with complete conversion to the high-spin state. Based on singular value decomposition, the authors proposed binding of three molecules of testosterone per molecule of P450 3A4 [39]. Spectrophotometric titrations of rabbit P450 1A2, another P450 that has been reported to show unusual substrate oxidation kinetics with very high cooperativity (Fig. 4) [41, 66, 67], have also revealed evidence of heterotropic cooperativity in binding of 1-alkoxynitrobenzene substrates in the presence of a Type II ligand, 1,4-phenylenediisocyanide [41, 67, 68]. These observations suggest that the substrate binding and dissociation may occur at different steps rather than taking place only at the initiation of the catalytic cycle as traditionally described. As a consequence of this, another degree of complexity is introduced to the P450-catalyzed oxidations (Fig. 1) which may explain the unusual oxidation kinetics observed with some P450s.

In addition to the above studies describing substrate binding of the ferric form of P450s, binding of coumarin to the reduced (ferrous) form of P450 2A6 was also demonstrated, albeit with a lower affinity than the ferric form [20]. A similar conclusion was reached when anaerobic spectrophotometric titration (for a description of techniques see [69-71]) of rabbit P450 1A2 with pyrene was carried out, with K_s values of 0.04 and 2.3 μM for the ferric and ferrous forms, respectively [41]. This change would be expected to lower the $E_{m,7}$ [32] by ~ 100 mV, assuming the accuracy of the low K_d value (although this case is complicated by the evidence for multiple ligand occupancy).

Steady-state spectrophotometric titrations are valuable in characterizing the thermodynamic aspects of substrate-P450 interactions; however, information regarding the individual step(s) involved in substrate binding is not readily accessible via these approaches. Pre-steady-state kinetic techniques, in particular stopped-flow spectrophotometry, are employed to discern the binding steps. In the case of some P450s including bacterial P450s 101A1 [9, 36] and 105D5 [37] or the mammalian P450 2A6 [20], the binding event is a single-step process as would be expected from a simple substrate-enzyme interaction. The k_{on} rates for these P450s are lower than but close to the second-order rates that would be expected from a diffusion-limited substrate-enzyme interaction (~10^7 to 10^9 M^{-1} s^{-1}, depending on the fraction of productive encounters) [32, 72]. Recently, we showed that the simple one-step $E + S \rightarrow ES$ type of substrate binding is not valid for all P450s, and the multi-step complex binding interactions observed with P450s 3A4 and 1A2 may have significant impact on the subsequent steps of the catalytic cycle, leading to the observed unusual substrate oxidation kinetics (i.e. cooperativity) [21, 35, 41, 68]. Furthermore, the rate of the substrate binding step that results in the absorbance-observable, low- to high-spin state change is significantly

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lower than the previously reported k_{on} rates for other P450s. This observation strongly suggests the presence of "absorbance-silent" steps prior to the displacement of water from the heme iron. Based on these observations, supported by our fluorescence studies (see below), we have proposed a multi-step binding model (Fig. 5) [21, 41] where a rapid initial interaction between the substrate and a peripheral site of the P450 is followed by movement of the substrate molecule(s) in the active site. The final steps are interpreted as conformational changes of the P450. Recent, evidence reported by other researchers provide further support for this general model [38, 73].

Fluorescence spectroscopy

Substrate binding by P450s has been studied using fluorescence spectroscopy, taking advantage of either the fluorescent properties of the substrate itself or the intrinsic fluorescence of the tryptophan and tyrosine residues of the P450s [74]. Fluorescent studies are important in studying substrate binding to those P450s that are isolated predominantly in the high-spin state (e.g. human P450 1A2 [67, 75]), as a tool to probe substrate interactions that do not involve heme, or to determine the amino acid residues that are involved in substrate binding. Depending on its orientation upon binding, the fluorescence emission of the substrate may be quenched via Förster resonance energy transfer (FRET) to the enzyme, in particular to the heme [76]. Fluorescence titration experiments based on the quenching of intrinsic tryptophan fluorescence have been used to determine the binding affinity of substrates to rabbit P450 1A2 [67]. Using excimer fluorescent properties of pyrene [74, 77, 78], evidence for binding of multiple pyrene molecules has been demonstrated for P450s 3A4 [79] and 1A2 [41]. Others have studied the binding of 1-pyrenebutanol to P450 107A1 [80] and P450 3A4 [42], showing that interaction of substrate with P450 quenches 1-pyrenebutanol fluorescence via FRET to the heme without having any impact on the iron spin state. In our own work, we used stopped-flow fluorescence quenching to study the binding of the fluorescent substrates bromocriptine and αNF to P450 3A4 [35] and pyrene and αNF to P450 1A2 [41]. During the course of these studies we demonstrated the presence of a rapid (close to diffusion-limited) binding step, which seems to occur at a peripheral site rather than close to heme, hence "absorbance-silent"[1]. The possible existence of a peripheral site on P450 3A4 is also supported by P450 3A4 crystal structures with progesterone and testosterone bound distant to the heme [81, 82].

Time-resolved fluorescence spectroscopy has been used to probe for conformational changes upon substrate binding to P450 101A1 [83, 84], P450 2D6 [85], and P450 3A4 [86, 87]. In these studies, fluorescence lifetime measurements (mainly that of tryptophan residues) are used together with FRET experiments to study conformational dynamics of P450s. Among the studied fluorescent P450 3A4 ligands are 2-*p*-toluidinylnaphthalene-6-sulfonic acid (TNS) (which fluoresces only in a highly hydrophobic environment [86, 88]), a synthetic deazaflavin-substituted testosterone analog [89, 90], and Nile Red [87, 91]. An alternate approach in studying substrate binding via fluorescence spectroscopy is the modification of the P450 of interest by a thiol-reactive fluorescent probe, which has been

[1]The term "absorbance silent" refers to a substrate-P450 interaction that does not involve a change in heme Soret spectra and therefore can not be detected by absorbance spectroscopy.

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applied to P450 107A1 [92] and P450 3A4 [93] in order to probe substrate-induced conformational changes. One advantage of this approach is the possibility of obtaining site-specific information regarding the substrate-P450 interactions depending on the positioning of the probe on the enzyme.

Nuclear magnetic resonance (NMR)

Changes in the NMR chemical shifts for specific residues can provide valuable information on the dynamics of substrate binding to proteins and associated conformational changes [94-96], one of the limitations being the necessity of isotopic labeling of the protein of interest [97, 98]. Earlier work (1-D and 2-D ^{1}H NMR) focused on substrate binding to P450 101A1, providing information on the structural features of the binding site [99-101], and the binding of tienilic acid, lauric acid, and diclofenac to P450 2C9 [102]. Binding of testosterone to P450 107A1 labeled with ^{15}N- phenylalanines (uniform–labeling) has been studied using 2-D ^{15}N- Heteronuclear single quantum coherence (HSQC) NMR [40, 103] in an attempt to understand better the substrate binding cooperativity observed for this P450 [104]. Solid-state deuterium magic angle spinning NMR [105-107] has been utilized to study binding of adamantine-d_{16} to P450 101A1 to measure the average distance between the deuteriums and the heme iron [108]. NMR spin-lattice relaxation T_1 rate measurements have also been used to calculate the distance between the substrate and the heme iron of P450s 1A1, 2B1 [109, 110], and 1A2 [111] and has served as a method to analyze the effects of binding of a substrate on the binding of another substrate to P450s 2C9 [112] and 3A4 [60, 113]. Recently, Yao *et al.* [73] reported their findings on camphor binding to P450 101A1 studied via T_1 relaxation measurements combined with ^{1}H-^{13}C HSQC studies of [$^{13}CH_3$]-threonine-labeled P450 101A1. Interestingly, their studies suggested that the camphor binds at a peripheral site in fast exchange at a location near the proposed entry channel. Solid-state 1-D and 2-D high-resolution NMR with selective labeling of the protein has been applied to the study of substrate binding to P450 102A1 [114, 115].

Electron paramagnetic resonance (EPR)

EPR spectroscopy can be applied to study directly the changes in the heme electronic environment, in particular the spin state upon substrate binding at cryogenic temperatures [116, 117]. Recently it was used to study the binding of multiple ligands to P450 3A4, providing a means of accurately quantifying the percentage of the low- and high-spin states without having to rely on extinction coefficients as in the case of absorbance titrations [38, 59].

Raman spectroscopy

Resonance Raman spectroscopy has been used to monitor conformational flexibility of the P450 heme as well as the spin state, based mainly on the vibrational modes of the porphyrin skeleton, heme side chains, and Fe-S stretching mode [118-122]. Substrate binding to P450s 102A1 [123], 19A1 (aromatase) [124], 2B4 [125], and 2D6 [126] has been studied with resonance Raman spectroscopy. Recently the differences between oxidation efficiency of two endogenous substrates of P450 21A1 (steroid 21-hydroxylase), progesterone and 17α-hydroxyprogesterone, have been explained with the aid of resonance Raman spectroscopy

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studies [127]. P450-substrate interactions have also been studied on self-assembled monolayer (SAM)-coated metal surfaces (silver and gold) by resonance Raman scattering spectroscopy [126, 128] where the sensitivity is increased via so called surface enhancement [129].

Surface plasmon resonance (SPR)

Localized surface plasmon resonance operates based on the principle of a shift in the wavelength of the scattering maximum upon binding of an analyte to nanoparticle surfaces and has been used to detect biomolecules including streptavidin [130] and anti-biotin [131]. Applicability of this approach to the study of small molecule-P450 interactions has been shown recently using camphor binding to P450 101A1 immobilized on SAM-coated Ag particles as a model system [132]. Surface plasmon resonance approach has been applied to P450 3A4 to study the binding of antifungal agents itraconazole and ketoconazole [133]. The kinetic binding studies had revealed evidence for a multi-step binding process with unexpectedly slow k_{on} rates for these molecules, an observation explained based on the theoretical studies describing the events involved in the diffusion of the substrate molecule from the surface into the active site of the enzyme. However, it should be pointed out that k_{on} rates for ligand binding are generally much slower in SPR than with solution methods (e.g., absorbance, fluorescence), possibly due to either slower instrument response time or to the effects of immobilizaiton [21].

Isothermal titration calorimetry (ITC)

Use of ITC in studying biomolecules has expanded rapidly as the sensitivity of the instruments has been improved significantly, with capability to detect a heat change as small as 0.1 μcal [134-137]. Since detection in ITC is based on heat changes, substrate binding to P450s can be studied without dependence on spectral changes, making ITC a valuable tool to probe for substrate binding at peripheral sites of P450s. Furthermore, a comprehensive picture of thermodynamics of substrate binding can be obtained with the estimation of enthalpic and entropic contributors to the overall free energy change [138]. A limitation has been the solubility of organic substrates in aqueous media and the low affinity for the enzyme for some substrates. During the course of our own work on the cooperativity of P450 3A4, we utilized ITC experiments to determine the stoichiometry of bromocroptine binding to P450 3A4 [21]. Muralidhara *et al.* have also employed ITC to study ligand binding to and resulting conformational changes of P450 2B4 [139, 140] and P450 107A1 [138], characterizing ligands by their “thermodynamic signatures”[2].

Circular dichroism (CD) and magnetic circular dichroism (MCD)

Circular dichroism has been used to detect conformational changes upon binding of substrates to P450 1A2, and binding of pyrene, 1-hydroxypyrene, αNF, and 1-isopropoxy-4-nitrobenzene-induced changes in the far-UV CD spectrum, consistent with a decrease in α-helicity of the enzyme [41, 68]. While CD spectroscopy is commonly used to analyze

[2]The term “thermodynamic signatures” is used by the authors to describe plots characterizing the thermodynamic parameters (free energy, enthalpy and entropy) associated with the binding of a particular ligand.

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secondary structures of P450s [141, 142], CD spectra collected in the presence of a magnetic field (magnetic CD) have more commonly served the purpose of studying ligation, oxidation, and spin state of the P450 heme [143-146]. Recently, substrate-free and -bound states of P450 101A1 have been characterized by MCD [147].

High pressure spectroscopy

The effect of substrate binding on the conformational features of the active site has been studied under high pressure, where hydration and compressibility become the major elements of the approach [148-150]. For these studies, the Soret band [151, 152] and the stretching mode vibration of CO bound to the reduced enzyme (monitored by IR spectroscopy) are utilized as spectral probes [153]. Also, flash photolysis [154, 155] and stopped-flow [150, 156] techniques have been employed under high pressure in order to investigate the steps involved in binding of substrates. Although much of the high pressure work has focused on bacterial enzymes P450 101A1 and P450 101A2, recently substrate binding to human P450 3A4 has also been investigated using high pressure spectroscopy [157, 158].

X-ray crystallography

The first x-ray crystallography work on P450s was published by Poulos and his associates and described the structure of camphor-bound P450 101A1 [159, 160] followed by the crystal structure of the free enzyme [161], raising questions around how the substrate gains access to the active site which is “inaccessible to the outside world” [162] (for reviews of P450 crysallography studies, see [5, 49]). Co-crystallization of P450 107A1 with the substrate molecule 6-oxyerythronolide B (the largest substrate molecule co-crystallized with a P450 at the time) revealed a rather large active site, paving the way for the modeling of mammalian P450s that are known to catalyze the oxidation of large substrates [163-165]. In fact, the homotropic cooperativity observed with P450 107A1 was rationalized by simultaneous binding of two molecules of androstenedione or 9-aminophenanthrene in the active site shown by x-ray crystallography [43] and proposed to be a model to explain the cooperative behavior observed with P450 3A4. More recent examples of simultaneous binding of two substrate molecules (flaviolin) came from the actinomycete P450s 158A1 [44] and P450 158A2 [45], both of which catalyze the dimerization of flaviolin. In the recent years, improvement of expression and crystallization methods has lead to the structural characterization of mammalian P450s, with x-ray crystal structures now available for eight P450s [47, 49, 166]. Among these P450s, substrate-bound crystal structures have been obtained for rabbit P450 2C5 with 4-methyl-*N*-methyl-*N*-(2-phenyl-2*H*-pyrazol-3-yl)benzenesulfonamide [167] and diclofenac [168], human P450 2C9 with warfarin [169] and flurbiprofen [170], human P450 2C8 with palmitic acid [171], human P450 2A6 with coumarin, indole, and nicotine [172, 173], rabbit P450 2B4 with bifonazole (actually a Type II ligand and not a substrate) [140], human P450 1A2 with αNF [174], and human P450 3A4 with progesterone [81], testosterone [82], and erythromycin [46]. Based on comparisons between substrate-free and -bound P450 structures, it can be concluded that substrate binding causes conformational changes, the extent of which is dependent on the nature of the substrate as well as the P450 enzyme involved. In this respect rabbit P450 2B4 appears

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to display the largest conformational changes upon ligand binding [140, 175], and substrate-bound crystal structures of P450s 2C5 [167] and 2A6 [172, 173, 176] suggest an "induced fit" for substrate binding. However, the structure of P450 2C8 seems much less flexible in binding retinoic acid and a variety of drugs. The ligand bound crystal structures of P450 3A4 has provided interesting information on this "drug-metabolizing" enzyme which can accommodate structurally diverse substrates [177]. Both the substrate bound and the free enzymes have large enough active sites to simultaneously accommodate two substrate molecules (e.g. testosterone), which may explain the unusual substrate oxidation kinetics observed with P450 3A4 [46, 81, 178]. Progesterone-bound P450 3A4 did not display any significant conformational changes [81] compared to the x-ray crystal structure of the free enzyme [178]. However, surprisingly, progesterone was determined to be bound at a peripheral site on the exterior of the protein rather than the active site [81]. This unexpected binding site may be viewed as an artifact and attributed to hydrophobic interactions between progesterone and the exterior of the enzyme. Another explanation, however, is the presence of a peripheral substrate recognition site where the initial interaction between the ligand and the enzyme takes place prior to substrate entry to the active site consistent with our proposed model of binding [21]. Similar observations of binding to peripheral sites have also been observed in the crystal structure of warfarin-bound human P450 2C9 [169], another enzyme which was shown to display cooperativity [179, 180] and palmitic acid-bound human P450 2C8 [171]. The first crystal structure of P450 3A4 with a substrate bound in the formal active site was reported by Ekroos *et al.* [46]. The erythromycin-bound crystal structure displayed a certain degree of conformational change with the apparent expansion and opening of the active site via the shift of the F helix providing further evidence for the plasticity of the enzyme as a means to accommodate diverse substrates with a wide range of molecular sizes. Another interesting observation in this work is the co-occupancy of the active site with two molecules of inhibitor ketoconazole [46], which may be interpreted as being consistent with multiple substrate binding as a mechanism for cooperative behavior.

Computational studies

With the availability of crystal structures of P450s, in particular human drug-metabolizing P450s, computational efforts had focused on predicting the P450s involved in the metabolism of drug molecules and the sites of oxidation [181-186]. These approaches are of interest in facilitating the solution of challenges faced in drug design and are used in the pharmaceutical industry. However, considering the complexity of P450 enzymes, with a significant degree of conformational flexibility and wide substrate diversity, these approaches have had limited success thus far in accurately predicting the site(s) of metabolism and the binding orientation(s) for a particular compound but may provide a source of information as a starting point for the biotransformation scientists [186, 187]. Molecular dynamic simulations have been applied to substrate binding to P450 3A4 in an attempt to explain cooperative behavior and results indicated the involvement of effector-substrate and effector-protein interactions [188]. Substrate binding to P450 2B4 has been studied via free energy calculations performed using density functional theory (DFT) and compared with spectral experimental studies [189]. The question raised by Poulos 20 years ago regarding the access of substrates to the active site, which is "inaccessible to the outside

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world" (see above), has been addressed via theoretical approaches [190]. Substrate access and product egress from the active site has been studied in detail by molecular dynamics and random expulsion molecular dynamics simulation methods [191-194]. Based on recent work on mammalian P450 2C5 and comparison with bacterial P450s, Schleinkofer *et al.* allude to the possibility of the existence of multiple pathways for substrate access and product egress which has been proposed to depend on the characteristic properties and function of a certain P450 [195].

Conclusion

Substrate binding to P450s has been studied extensively and continues to be an area of major interest. Despite many technological advances in instrumentation, the availability of high-resolution x-ray crystal structures and informative results arising from detailed studies, many questions still remain regarding the P450s and in particular the substrate binding step, the focus of the present review. As presented above, there are more exceptions than rules when it comes to the binding of a substrate to different P450s. The mechanisms underlying cooperative substrate oxidation kinetics are still not completely understood although many proposals have been presented. The conformational flexibility observed with a number of P450s have arisen many additional questions, making the prediction of substrate properties challenging from a drug discovery point of view. However, it is important to realize the progress which has been made since the first binding spectra obtained over forty years ago [22, 196].

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Abbreviations

CD	Circular dichroism
DFT	Density functional theory
EPR	Electron paramagnetic resonance
FRET	Förster resonance energy transfer
HSQC	Heteronuclear single quantum coherence
ITC	Isothermal titration calorimetry
NMR	Nuclear magnetic resonance
MCD	Magnetic circular dichroism
αNF	α-Naphthoflavone
P450	Cytochrome P450 (also termed "heme thiolate P450")
SAM	Self-assembled monolayer
SPR	Surface plasmon resonance

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TNS 2-*p*-Toluidinylnaphthalene-6-sulfonic acid

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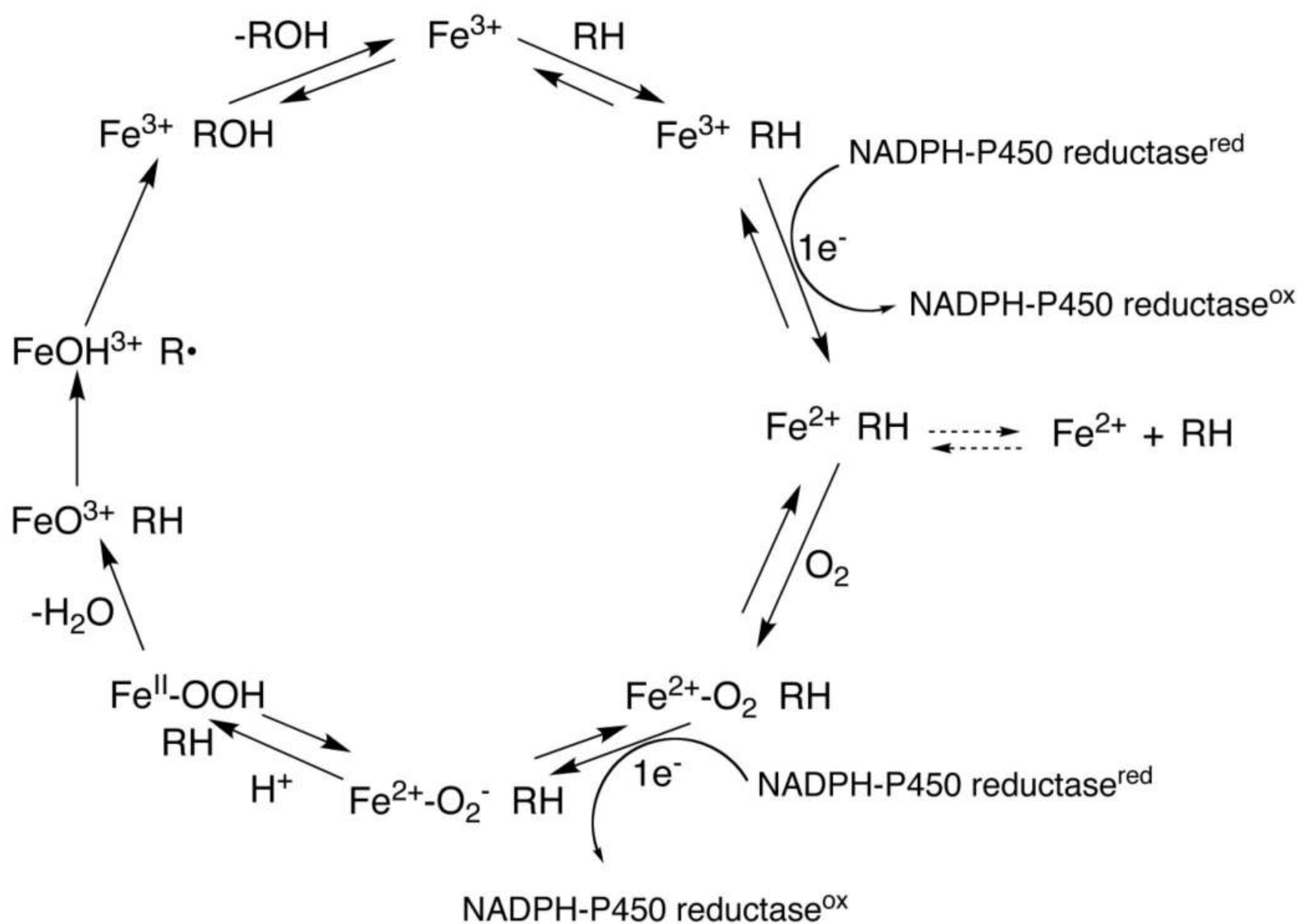

Fig. 1. Generalized P450 catalytic cycle

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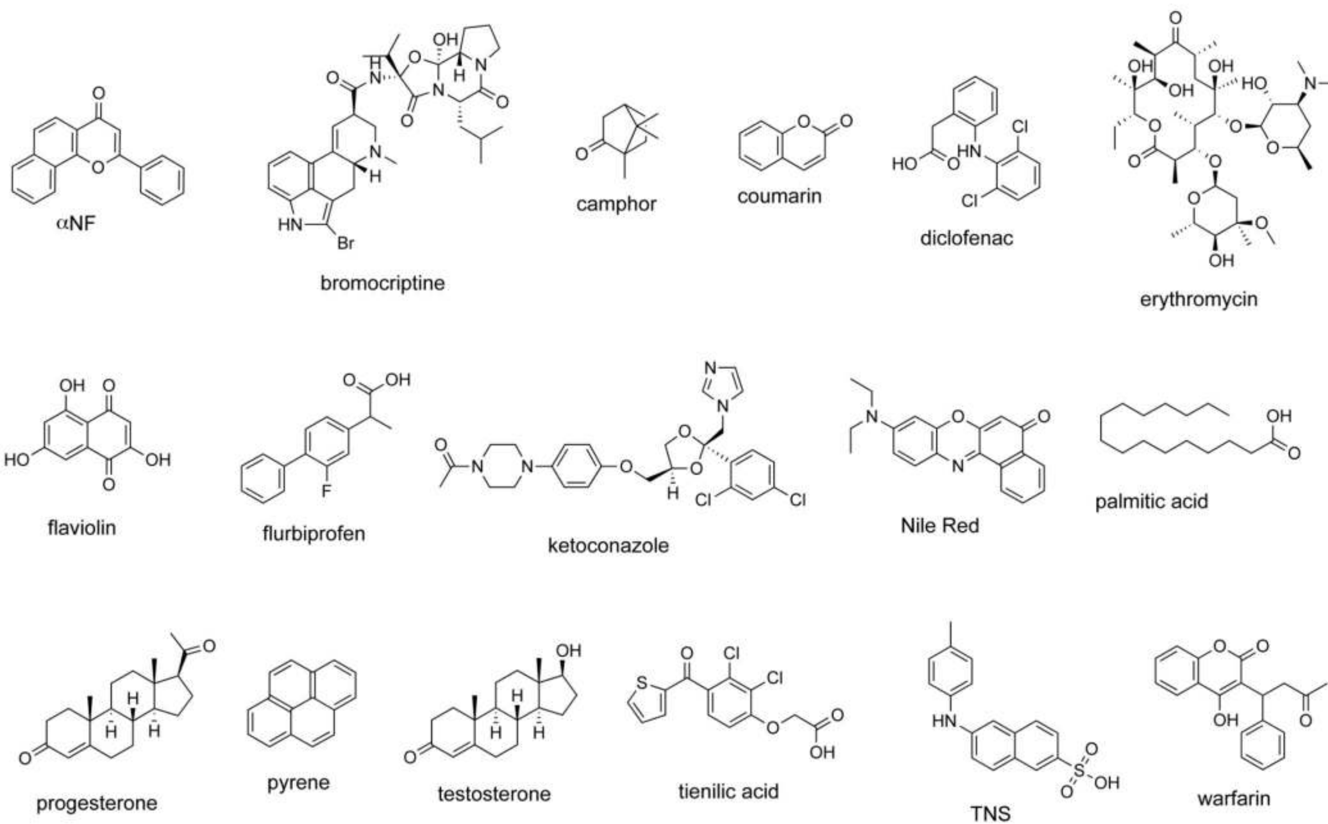

Fig. 2. Structures of selected ligands described in the text

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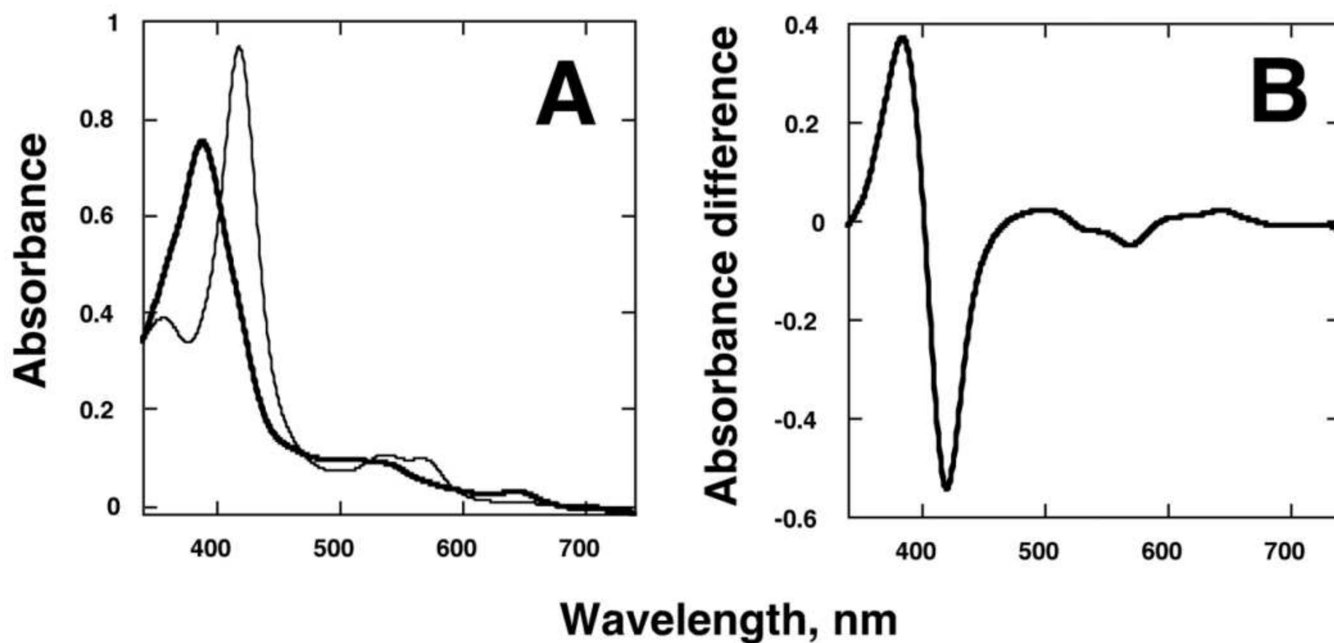

Fig. 3. Spectra of P450 2A6 complexes

A, Spectra were recorded in 50 mM potassium phosphate buffer (pH 7.4) with 5.2 μM P450 2A6, either without (—) or with (—) 50 μM coumarin. *B*, Difference spectrum obtained by mathematically subtracting the spectrum of the unbound P450 from the bound. Reprinted with permission from [20].

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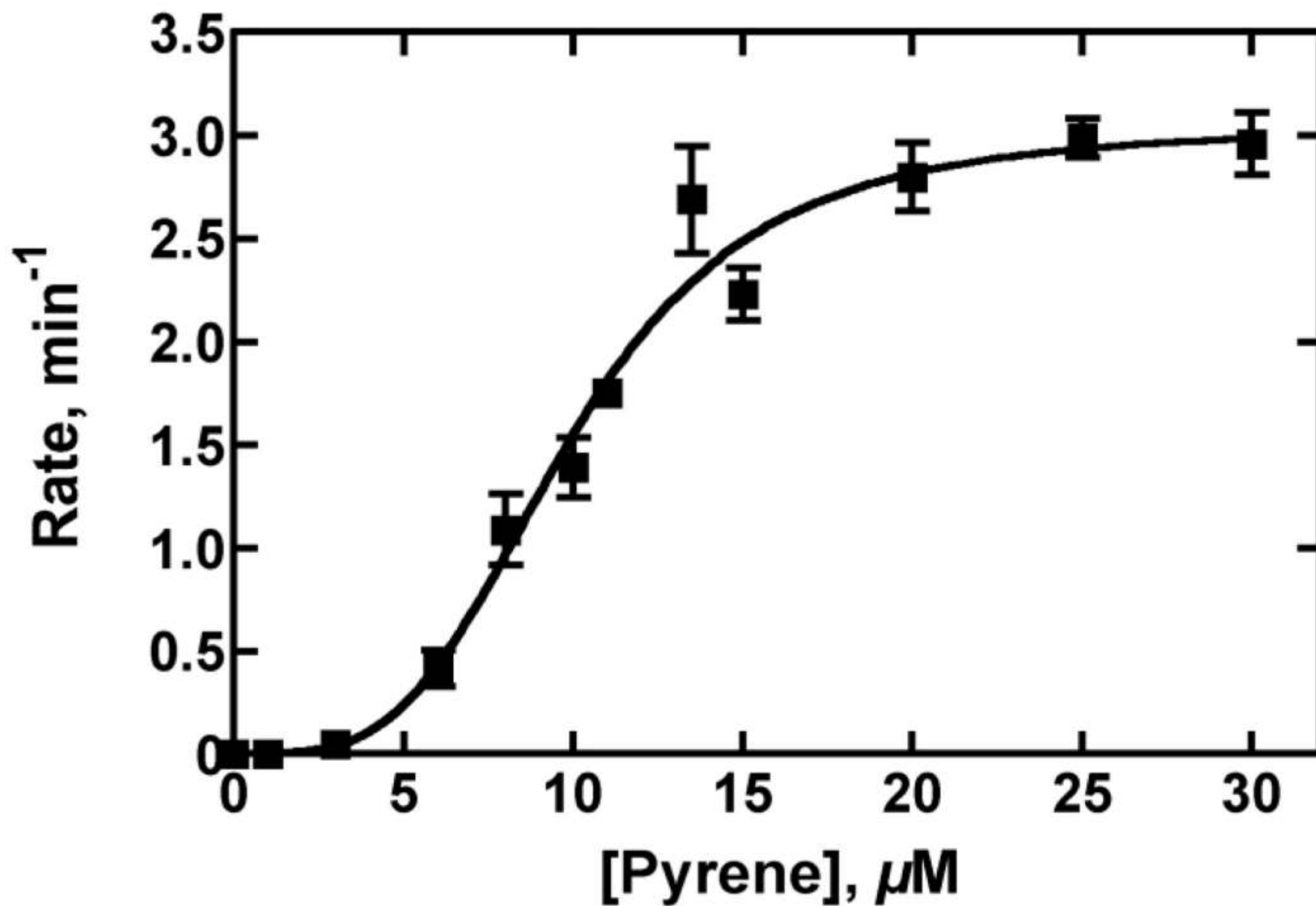

Fig. 4. Steady-state kinetics of oxidations catalyzed by P450 1A2

Pyrene 1-hydroxylation; data points are set to the equation $v = k_{cat} \cdot S^{n} (S_{50}^{n} + S^{n})^{-1}$, with $k_{cat} = 3.0 \pm 0.1$ min^{-1}, $n = 3.6 \pm 0.6$ and $S_{50} = 9.9 \pm 0.5$ μM. Reprinted with permission from [41].

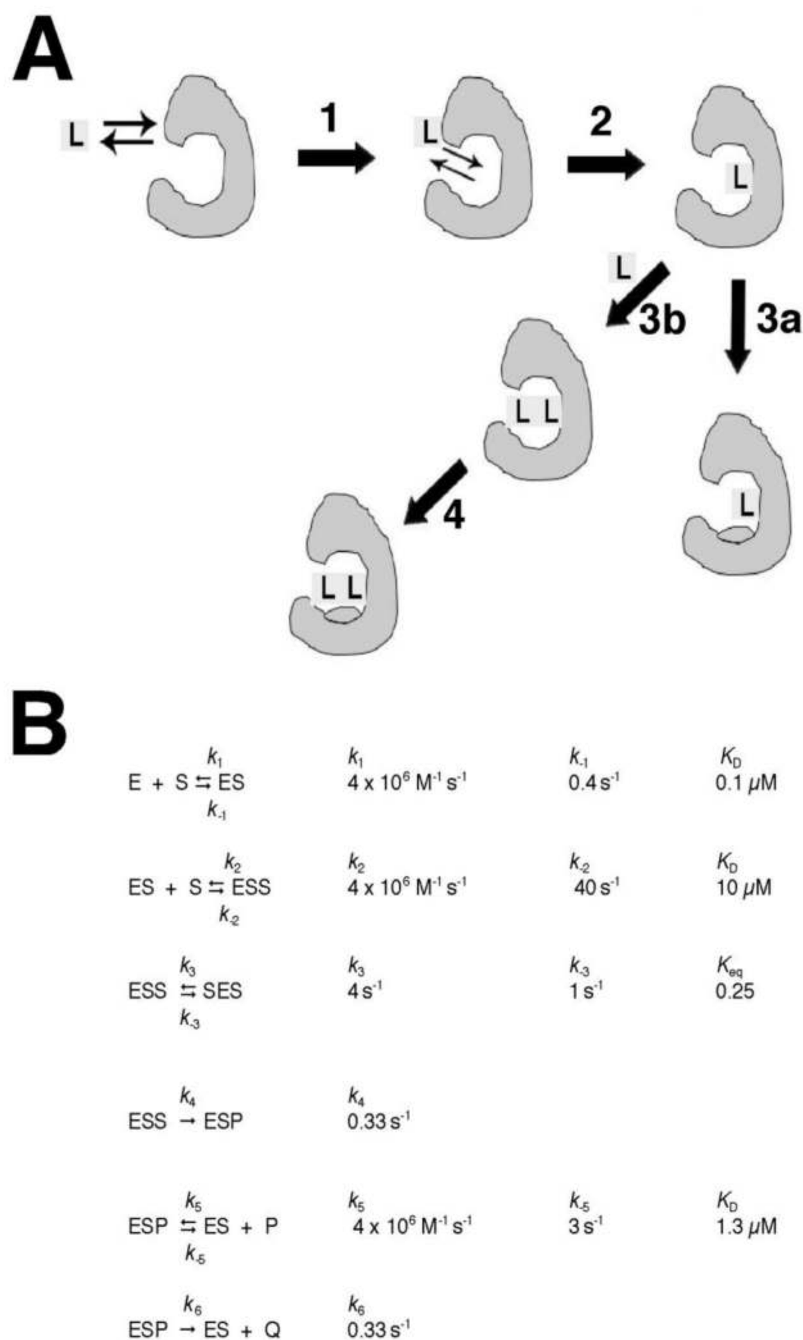

Fig. 5. *A*, Scheme depicting proposed events in ligand binding to ferric P450 1A2

See text and original reference [41] for more discussion. *Step 1:* The ligand L first interacts with P450 1A2 at a peripheral site. *Step 2*: L is translocated to the interior of the protein, *Step 3a*: A conformational change in the P450 occurs. *Step 3b*: If L is small enough for two molecules (of L) to occupy the active site, a second molecule of L can enter the active site. *Step 4*: Conformational change of the P450. ***B*, Model and rate constants used in fitting.** E: P450 1A2, S: pyrene, P: 1-hydroxypyrene, Q: dihydroxypyrene products (1,5-, 1,6-, and 1,8-). The kinetic scheme involves sequential binding of two pyrene molecules (k_1, k_{-1}, k_2,

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k_{-2}),a conformational change (k_3, k_{-3}),, oxidation of pyrene only in the complex with two pyrenes (k_4, k_{-4}), release of 1-hydroxypyrene (k_5, k_{-5}), and conversion of 1-hydroxypyrene to dihydroxypyrene(s) from the binary complex (k_6, k_{-6}). Reprinted with permission from [41].

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Table 1

Comparison of different approaches used to study substrate binding to P450s 3A4 and 1A2

	Steady state absorbance	Steady state fluorescence	Stopped-flow absorbance	Stopped-flow fluorescence	Circular dichroism	EPR	X-ray crystallography – homology modeling
Testosterone – P450 3A4	Cooperative binding [34, 58]		Multi-step binding [21]			Cooperative binding, high affinity non productive binding site [59]	Possible peripheral binding site (x-ray) [82]
αNF – P450 3A4	Cooperative binding [34], high affinity “absorbance silentȍ binding site [38]		Multi-step binding [21]	Multi-step binding with rapid “absorbance silent” first step [21]		High affinity “absorbance silent” binding site [38]	
αNF – P450 1A2	Non-cooperative binding [41]		Multi-step binding [41]	Multi-step binding with rapid “absorbance silent” first step [41]	Ligand induced conformational change of P450 [41]		
Pyrene – P450 1A2	Non-cooperative ligand binding [41]	Possible simultaneous occupancy of the active site with two pyrene molecules [41] (also for P450 3A4 [79])	Multi-step binding [41]	Multi-step binding with rapid “absorbance silent” first step [41]	Ligand induced conformational change of P450 [41]		Possible simultaneous occupancy of the active site with two pyrene molecules (homology modeling) [41]
P450				Substral			Inhibitors

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Table 2

Summary of x-ray crystal structures of mammalian P450s bound to substrate or inhibitors mentioned in the text.

Human P450 3A4	Progesterone [81], erythromycin [46], testosterone [82]	Ketoconazole [46], metyrapone [81]
Human P450 2A6	Coumarin, indole, nicotine [172, 173]	Methoxsalen [172]
Human P450 1A2	α-Naphthoflavone [174]	
Rabbit P450 2B4		Bifonazole [140]
Rabbit P450 2C5	4-methyl-*N*-methyl-*N*-(2-phenyl-2*H*-pyrazol-3-yl)benzenesulfonamide [167], diclofenac [168]	
Human P450 2C8	Palmitic acid [171]	
Human P450 2C9	Warfarin [169], flurbiprofen [170]	